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The bovine innate immune response during experimentally-induced *Pseudomonas aeruginosa* mastitis

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Abstract

Almost half of all clinical cases of mastitis are caused by Gram-negative bacteria. Among these bacteria, intramammary infection by *Pseudomonas aeruginosa* remains one of the most refractory to antibiotic therapy. The ability to recognize potentially harmful pathogens whether previously encountered or not, as well as the induction of an initial pro-inflammatory response to these pathogens, are critical components of host innate immunity. Although the innate immune response to another Gram-negative mastitis-causing pathogen, *Escherichia coli*, has been well-characterized, little is known about the response to other Gram-negative bacteria, including *P. aeruginosa*. The objective of the current study was to characterize the systemic and localized bovine innate immune response to intramammary infection with *P. aeruginosa*. The contralateral quarters of ten mid-lactating Holstein cows were challenged with either saline or *P. aeruginosa*. Following the establishment of infection, milk samples were collected and assayed for changes in cytokine and growth factor concentrations, complement activation, and changes in the levels of soluble CD14 (sCD14) and lipopolysaccharide (LPS)-binding protein (LBP), two accessory molecules involved in host recognition of Gram-negative bacteria. Initial increases in milk somatic cell counts were evident within 12 h of experimental challenge and remained elevated for $\geq$3 weeks. Increased permeability of the mammary gland vasculature, as evidenced by elevated milk levels of BSA, was initially observed 20 h post-infection and persisted for 2 weeks. Within 32 h of challenge, increased levels of IL-8, TNF-α, IL-10, and IL-12 were detected, however, the elevated levels of these cytokines were not sustained for longer than a 24 h period. In contrast, elevations in IL-1β, IFN-γ, TGF-α, TGF-β1, TGF-β2, sCD14, LBP, and activated complement factor 5 (C5a) were sustained for periods of >48 h. Systemic changes were characterized by elevated body temperature, induction of the acute phase protein synthesis of serum amyloid A and LBP, and a transient decrease in circulating neutrophils and lymphocytes. Together, these data demonstrate the capability of the mammary gland to mount a

Abbreviations: LBP, LPS-binding protein; LPS, lipopolysaccharide; mCD14, membrane-bound CD14; SAA, serum amyloid A; SCC, somatic cell count; sCD14, soluble CD14; TLR, toll-like receptor

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robust innate immune response to *P. aeruginosa* that is characterized by the induction of pro-inflammatory cytokines, complement activation, and increased levels of accessory molecules involved in Gram-negative bacterial recognition.
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Keywords: Bovine; Cytokine; Endotoxin; Innate immunity; *Pseudomonas aeruginosa*; Mastitis

1. Introduction

Mastitis is the most prevalent and costly production disease in dairy herds worldwide and most commonly develops in response to intramammary bacterial infection (Schalm et al., 1971; Seegers et al., 2003). Although improved dairy herd practices have been successful at controlling many of the contagious Gram-positive pathogens that cause mastitis, these practices have largely been ineffective at reducing the incidence of intramammary infections caused by Gram-negative bacteria (Jackson and Bramley, 1983; Erskine et al., 1991; Hillerton et al., 1995). Approximately 40% of clinical cases of mastitis are caused by Gram-negative bacteria and nearly 25% of these cases result in death or culling of the animal (Eberhart, 1984; Erskine et al., 1991). As dairymen continue to strive for lower bulk tank milk somatic cell counts (SCC), economic losses attributed to intramammary Gram-negative infection are expected to increase because of the inverse relationship between the incidence of these infections and bulk milk SCC (Barkema et al., 1998).

The most prevalent Gram-negative bacteria that induce mastitis in cattle are *Escherichia coli* and much of our understanding of the immune response to Gram-negative infection is derived from studies with these bacteria (Shuster et al., 1995, 1997; Hirvonen et al., 1999; Lee et al., 2003a,b). In contrast, much less is known about the mammary gland innate immune response to other prevalent Gram-negative bacteria, including *Pseudomonas aeruginosa*. Of the Gram-negative bacteria that cause mastitis, *P. aeruginosa* has been reported to be among the three most prevalent cultured from bovine milk samples and the three most frequently associated with clinical mastitis (Howell, 1972; Wilson et al., 1997; Barkema et al., 1998).

Clinical mastitis resulting from herd outbreaks of *P. aeruginosa* has been reported to be caused by a variety of factors, including contaminated dry-off preparations (Anderson et al., 1979; Nicholls et al., 1981; Osborne et al., 1981), teat wipes (Daly et al., 1999; Power, 2003), and wash water used to clean udders prior to milking (Curtis, 1969; Malmo et al., 1972; Kirk and Bartlett, 1984; Erskine et al., 1987). The minimal nutritive requirements of *P. aeruginosa*, its ability to readily grow in soil and water, and its relative resistance to chemical disinfectants all make eradication of this pathogen from the cow's environment virtually impossible (Howell, 1972; Packer, 1977). The clinical course of cows with a *P. aeruginosa* intramammary infection is initially characterized by the development of acute mastitis that often develops into a state of chronic infection (Howell, 1972; Packer, 1977; Power, 2003). Acute gangrenous mastitis has been reported to develop in approximately 10% of all cases and systemic complications resulting in death of the animal are not uncommon (Howell, 1972; Malmo et al., 1972; Packer, 1977; McLennan et al., 1997; Power, 2003). In general, therapeutic treatment of intramammary infections caused by Gram-negative bacteria remains suboptimal and among these bacteria, *P. aeruginosa* is the most resistant to conventional antibiotics (Huber, 1977; McDonald et al., 1977).

The pathogenicity of *P. aeruginosa* is governed by a variety of virulence factors (Van Delden and Iglewski, 1998; Lyczak et al., 2000). Bacterial lipopolysaccharide (LPS), a component of the outer leaflet of all Gram-negative bacteria including *P. aeruginosa*, protects against the lytic effects of complement, confers resistance to phagocytosis, and facilitates colonization (Lerouge and Vanderleyden, 2002). Other virulence factors include pili and flagella, which both mediate bacterial adherence and the latter of which also confers motility (Van Delden and Iglewski, 1998; Lyczak et al., 2000). Local tissue damage caused by bacterial secretion of exotoxin A, exoenzyme S, and elastase facilitate *P. aeruginosa* invasion and dissemination. *P. aeruginosa*-derived elastase is also reported to inhibit monocyte and neutrophil chemotaxis and respiratory burst (Kharazmi and Nielsen, 1991; Ijiri et al., 1994). *P. aeruginosa* are also able to form a biofilm through secretion of the exopolysaccharide alginate (Van

Delden and Iglewski, 1998). This surrounding matrix protects the bacteria from leukocyte-mediated phagocytosis and complement activation, and confers resistance to antibiotics and disinfectants. Together, these virulence factors contribute to the ability of *P. aeruginosa* to establish infection and resist host innate immune defenses.

Two critical components of host innate immunity are pathogen detection and the ability to mount a pro-inflammatory response (Uthaisangsook et al., 2002). Detection of infectious pathogens is mediated by the expression of evolutionarily conserved pattern recognition receptors that are capable of recognizing common bacterial motifs shared by diverse pathogens (Akira et al., 2001). The toll-like receptors (TLR) are a highly conserved family of pattern recognition receptors involved in pathogen detection. Bacterial LPS contains motifs that are recognized by TLR-4. Presentation of LPS to this receptor is mediated by the accessory molecules CD14 and LPS-binding protein (LBP). TLR-4 has also been implicated in the recognition of bacterial-derived elastase and exoenzyme S (Devaney et al., 2003; Epelman et al., 2004). The recognition of other *P. aeruginosa* components is mediated by other TLR family members (Akira, 2003). TLR pairs, including TLR 1 and 2 and TLR 2 and 6, recognize lipopeptides, whereas, individual TLR's, such as TLR-5 and TLR-9, recognize flagellin and unmethylated CpG DNA (Akira, 2003; Adamo et al., 2004; Greene et al., 2005). In addition, other pattern recognition molecules, such as mannose-binding lectin, undoubtedly contribute to host recognition of *P. aeruginosa* (Kuipers et al., 2003).

Subsequent to pathogen detection is the activation of intracellular signaling pathways leading to cytokine production (Akira et al., 2001). Cytokines, in turn, contribute to the many facets of the inflammatory response including: (1) the febrile response; (2) the induction of hepatic synthesis of acute phase proteins; (3) leukocyte recruitment; and (4) changes in vascular permeability, tone, and activation (Dinarello, 1996; Thijs et al., 1996). Cytokines also function to link the innate and adaptive branches of immunity, as well as to contribute to the resolution of inflammation (Spits and de Waal Malefyt, 1992; Trinchieri, 1997; Ma, 2001; Redpath et al., 2001).

Although much is known about the innate immune response to *E. coli*, little is known about the mammary gland innate immune response to other Gram-negative bacteria that cause mastitis, including *P. aeruginosa*. Whether different Gram-negative bacteria elicit a similar inflammatory response, perhaps by virtue of the expression of conserved immunostimulatory components remains unknown. Therefore, the objective of this study was to characterize the bovine innate immune response to intramammary infection with *P. aeruginosa*, a less well-characterized, but clinically important, mastitis-causing Gram-negative pathogen.

2. Materials and methods

2.1. Cows

Ten healthy, mid-lactating Holstein cows (209 ± 34 days in milk) were selected on the basis of milk SCC of <200,000 cells/mL and the absence of detectable bacteria growth from three daily consecutive aseptic milk samples plated on blood agar plates. Mean (±S.E.) milk SCC for all bacterial-challenged quarters at the start of the study were 38,600 ± 13,171 cells/mL. The use and care of all animals in this study was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

2.2. Intramammary challenge with P. aeruginosa

Prior to intramammary challenge, 10 mL of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Sparks, MD) were inoculated with a strain of *P. aeruginosa* originally isolated from a clinical case of mastitis (gift of Dr. W.D. Schultze, USDA-ARS Beltsville Agricultural Research Center, Beltsville, MD). Following a 6 h incubation at 37 °C, 1 mL of the culture was transferred to an aerating flask containing 99 mL of tryptic soy broth and incubated overnight at 37 °C and 225 rpm. After incubation, the flasks were placed in an ice water bath and mixed by swirling. A 1 mL aliquot from the flask was serially diluted in PBS and 0.1 mL of the resulting dilutions spread on trypticase soy agar plates containing 5% bovine blood and 0.1% esculin (Becton-Dickinson Diagnostic Systems, Inc., Sparks, MD). The plates were incubated overnight at 37 °C while the aerating flasks containing the stock cultures were maintained at 4 °C. After determining the concentration (CFU/mL)

of the stock cultures based on the spread plate colony counts, the stock culture was diluted in PBS to yield a final approximate concentration of 100 CFU/mL.

Immediately following the morning milking, the contralateral quarters of each animal were infused with 2 mL of either the final dilution of *P. aeruginosa* or PBS alone. To determine the actual number of bacteria infused, serial dilutions of the challenge innoculum were plated and incubated overnight. The number of bacteria infused into each quarter was determined to be 115 CFU. Throughout the study, aseptic milk samples were collected from control and infected quarters and plated on blood agar plates. Colonies with a characteristic blue-green discoloration due to the production of pyocyanin and that contained Gram-negative, oxidase positive rods were considered positive for *P. aeruginosa* (Koneman et al., 1997). Confirmatory identification was performed by the Maryland Department of Agriculture Animal Health Section Laboratory (College Park, MD).

2.3. Determination of milk somatic cell and circulating differential white blood cell counts

To quantitate somatic cells, milk samples were heated to 60 °C and subsequently maintained at 40 °C until counted on an automated cell counter (Fossomatic model 90, Foss Food Technology, Hillerod, Denmark) as previously described (Miller et al., 1986). For the determination of circulating differential white blood cell counts, tail vein blood samples were collected in Vacutainer® glass tubes containing K_3 EDTA (Becton-Dickinson Corp, Franklin, Lakes, NJ), inverted ×10, placed on a rocker for 15 min, and analyzed using a HEMAVET® 3700 automated multispecies hematology system (CDC Technologies, Inc., Oxford, CT).

2.4. Whey and plasma preparation

For the preparation of whey, milk samples were centrifuged at 44,000 × *g* at 4 °C for 30 min and the fat layer removed with a spatula. The skimmed milk was decanted into a clean tube and centrifuged again for 30 min as above and the translucent supernatant collected and stored at −70 °C. For the preparation of plasma, tail vein blood samples were collected as above, inverted ×10, centrifuged at 1500 × *g* for 15 min, and the clear plasma supernatant collected, aliquotted, and stored at −70 °C.

2.5. Enzyme-linked immunosorbent assays (ELISA's)

ELISA's for BSA, C5a, IFN-γ, IL-1β, IL-8, IL-10, IL-12, LBP, sCD14, and TNF-α, were all performed as previously described (Bannerman et al., 2004). For the determination of serum amyloid A (SAA) concentra-

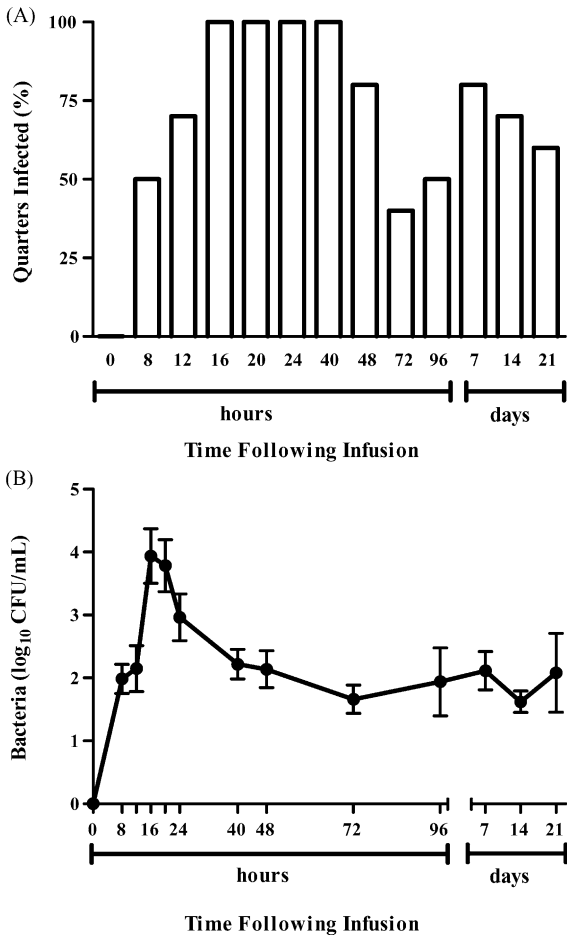

Fig. 1. Intramammary growth of *P. aeruginosa* following experimental challenge. Following intramammary infusion of 115 CFU of *P. aeruginosa* into one quarter on each of 10 cows, sterile milk samples were collected from all infused quarters at various time points and plated. The percent of quarters in which viable *P. aeruginosa* were recovered is indicated (A). In those quarters where *P. aeruginosa* were recovered, the mean (±S.E.) of $\log_{10}$ CFU/mL is shown (B).

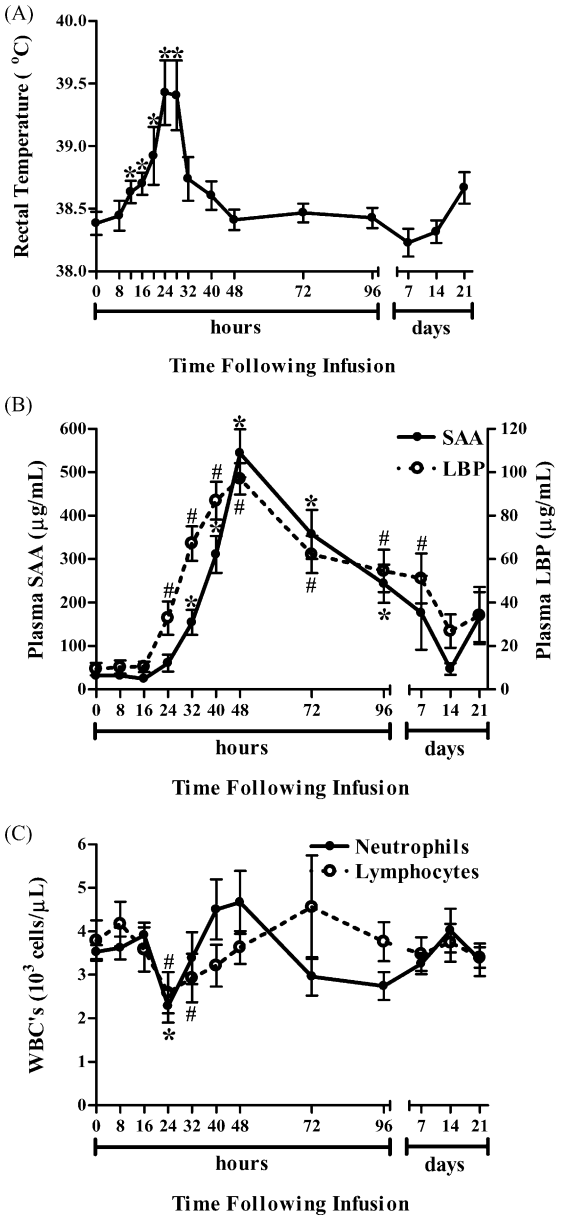

Fig. 2. The systemic effects of intramammary *P. aeruginosa* infection. As indicators of the systemic response to *P. aeruginosa* intramammary infection, changes in rectal temperature (A), induction of acute phase synthesis of SAA and LBP (B), and alterations in circulating leukocyte populations were studied (C). Mean (±S.E.) rectal temperature is reported in °C (A). (*) Significantly increased compared to time 0 ($P < 0.05$). Blood samples were collected immediately prior to and for various time points following intramammary infection and plasma assayed for SAA and LBP by ELISA (B). Mean (±S.E.) levels of plasma SAA and LBP are reported in µg/mL. (*, #) Significantly increased SAA and LBP levels, respectively, compared to time 0 ($P < 0.05$). Differential neutrophil and lymphocyte counts were determined in whole blood obtained in parallel as above (C). Mean (±S.E.) cell counts are reported in thousands/µl. (*, #) Significantly decreased circulating neutrophils or lymphocytes, respectively, compared to time 0 ($P < 0.05$).

tions, samples were diluted 1:4000 and assayed with a commercially available kit (Tri-Delta Diagnostics, Inc., Morris Plains, NJ) as previously described (Nielsen et al., 2004). Milk levels of TGF-α, TGF-β1, and TGF-β2 were quantified using commercially available kits (R&D Systems, Inc., Minneapolis, MN) that have been previously validated for use with bovine milk samples (Ginjala and Pakkanen, 1998; Pakkanen, 1998). Samples assayed for TGF-β1 were first activated by incubating undiluted whey with an equal volume of an aqueous solution containing 2.5N acetic acid and 10 M urea for 10 min. The reaction was then neutralized by the addition of a half volume of an aqueous solution containing 2.7N sodium hydroxide and 1 M HEPES and the final reactants diluted four-fold with the supplied diluent. Samples assayed for TGF-β2 were first diluted (1:13) in deionized water and subsequently activated according to the manufacturer's instructions.

2.6. Statistical methods

Repeated measures ANOVA was performed using the PROC MIXED model (SAS 8.2; SAS Institute, Cary, NC) to compare the mean responses between experimental groups and the pre-infused (time 0) groups. For statistical analysis of milk SCC, data were transformed to $\log_{10}$ values. A P-value of <0.05 was considered significant.

3. Results

3.1. Recovery of viable bacteria from challenged quarters

To determine whether the initial intramammary infusion of 115 CFU of *P. aeruginosa* resulted in the establishment of infection, aseptic milk samples were collected at varying time points following infusion, plated, and colonies counted. Within 16 h of and up to 40 h following challenge, viable *P. aeruginosa* were

recovered from all ten challenged quarters (Fig. 1A). Six of the 10 quarters remained infected throughout the end of the study. Maximal numbers of *P. aeruginosa* (3.94 ± 0.43 $\log_{10}$ CFU/mL) were recovered from milk samples within 16 h of infusion, after which they declined until 40 h and then remained relatively constant throughout the end of the study (Fig. 1B).

3.2. Acute phase systemic response to intramammary infection with P. aeruginosa

To determine whether *P. aeruginosa* intramammary infection could elicit a systemic response, changes in body temperature, acute phase protein synthesis, and differential white blood cell counts were assayed. Elevated body temperatures were first observed 12 h post-infection, reached a peak of 39.43 ± 0.26 °C at 24 h, and returned to baseline levels by 32 h (Fig. 2A). The systemic response to infection was further characterized by the induction of acute phase synthesis of SAA and LBP (Fig. 2B). Circulating levels of LBP and SAA initially increased within 24 and 32 h of infection, respectively, and reached maximal levels by 48 h. Within 7 and 14 days, respectively, plasma concentrations of SAA and LBP returned to pre-challenge (time 0) levels. Changes in circulating levels of SAA and LBP strongly correlated with one another ($r = 0.9005$). In addition to elevations in body temperature and the induction of acute phase protein synthesis, intramammary infection with *P. aeruginosa* elicited a transient decrease in circulating levels of neutrophils and lymphocytes, the most predominant white blood cells, within 24 h (Fig. 2C).

3.3. Intramammary infection with P. aeruginosa is characterized by elevations of milk SCC

Relative to pre-challenged (time 0) quarters, increased milk SCC were evident within 12 h of *P. aeruginosa* infection and remained elevated for ≥21 days (Fig. 3). Maximal elevations in milk SCC were observed 32 h post-infusion and reached a level of $62.92 \times 10^6 \pm 6.39 \times 10^6$ cells/mL. We have previously established that the actual infusion process, itself, does not induce an inflammatory response as measured by changes in milk SCC and cytokine

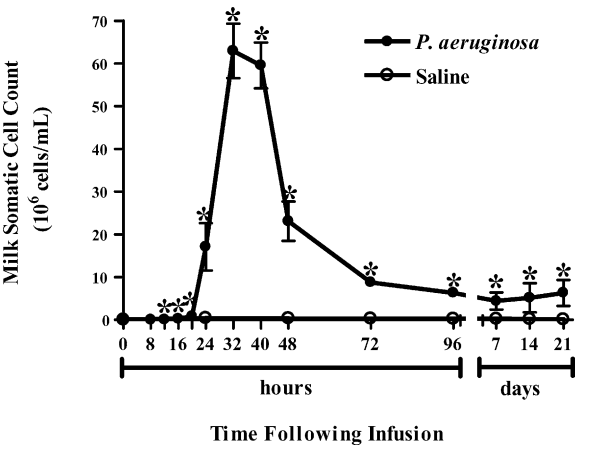

Fig. 3. Effect of intramammary infection with *P. aeruginosa* on milk somatic cell counts (SCC). Somatic cell counts were determined in milk samples obtained immediately prior to and for various time points following intramammary infusion of either saline (mock-infected) or *P. aeruginosa*. Mean (±S.E.) milk SCC are reported in millions/mL. (*) Significantly increased in *P. aeruginosa* infected quarters relative to time 0 ($P < 0.05$).

expression (Bannerman et al., 2003, 2004). To confirm that the infusion process did not elicit an inflammatory response in the current study, milk samples were obtained from saline-infused (i.e., mock-infected)

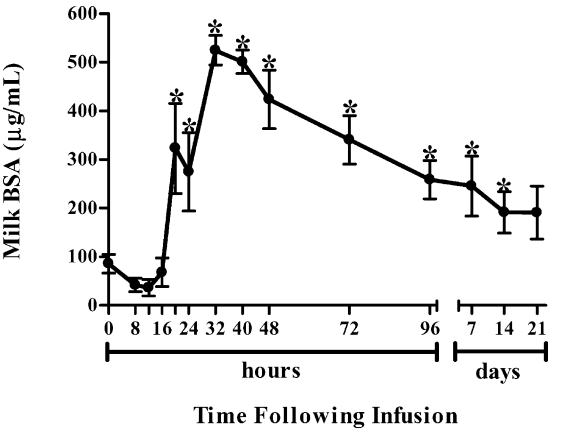

Fig. 4. Effect of intramammary infection with *P. aeruginosa* on mammary vascular permeability. As an indicator of change in mammary gland vascular permeability, BSA levels were measured by ELISA in milk samples collected immediately prior to and for various time points following intramammary infusion of *P. aeruginosa*. Mean (±S.E.) BSA levels are reported in μg/mL. (*) Significantly increased compared to time 0 ($P < 0.05$).

quarters every 24 h during the first 4 days and on a weekly basis thereafter, and milk SCC determined (Fig. 3). In contrast to the *P. aeruginosa*-infected quarters, milk SCC in saline-infused quarters remained unchanged throughout the study.

3.4. Breakdown in the integrity of the blood-mammary gland barrier during intramammary infection with P. aeruginosa

To determine whether intramammary infection with *P. aeruginosa* could alter mammary gland vasculature permeability, milk BSA levels were assayed by ELISA. Milk from quarters infused with *P. aeruginosa* demonstrated an acute increase in levels of BSA within 20 h of infection that persisted for an additional 2 weeks (Fig. 4). Peak levels of BSA were observed within 32 h of challenge and reached a concentration of 524.94 ± 30.27 μg/mL.

3.5. Changes in milk complement activation and pro-inflammatory cytokine levels during the course of P. aeruginosa intramammary infection

Increased milk levels of the chemoattractants C5a and IL-8 were observed within 20 h of infection (Fig. 5A). Elevated levels of C5a were sustained for an additional 52 h, whereas those of IL-8 returned to baseline levels within 12 h of the initial increase. Maximal levels of IL-8 (90.47 ± 46.61 pg/mL) and

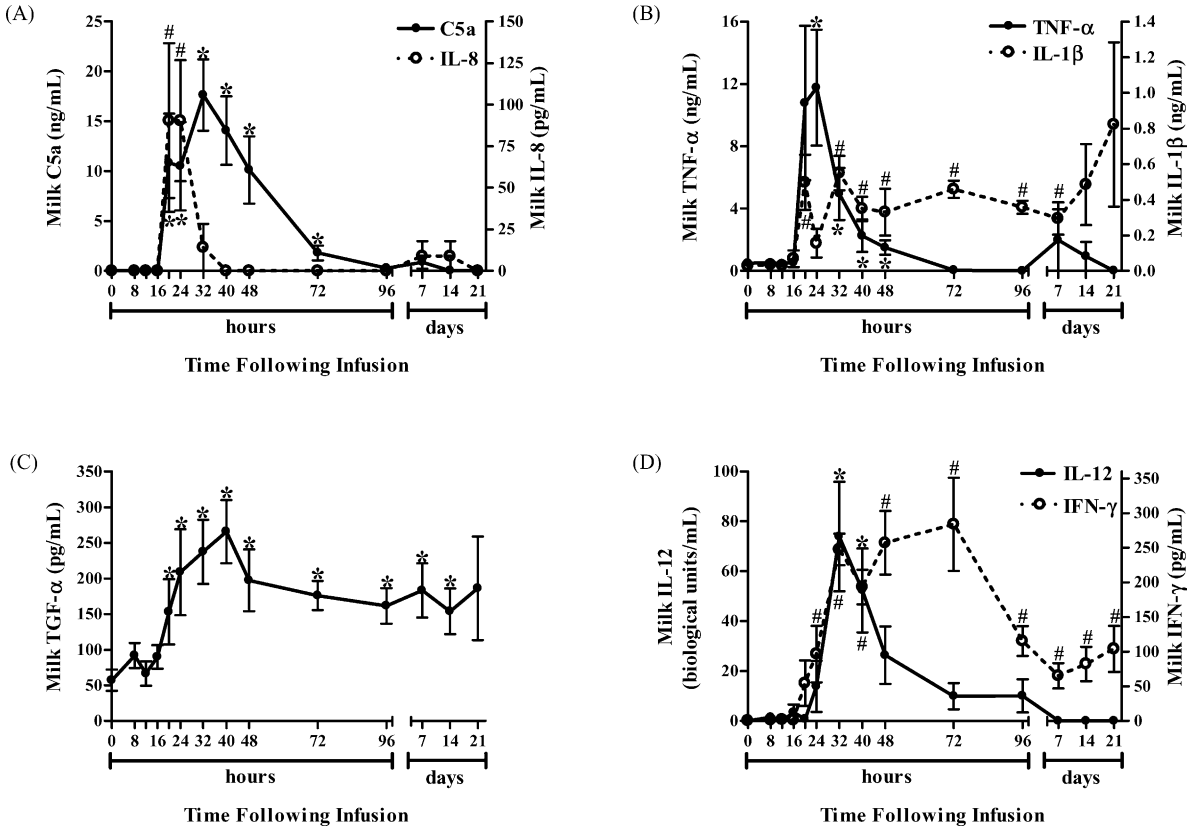

Fig. 5. Effect of intramammary infection with *P. aeruginosa* on complement activation and pro-inflammatory cytokine levels in milk. Milk samples collected immediately prior to and for various time points following intramammary infection with *P. aeruginosa* were analyzed by ELISA for the following: C5a and IL-8 (A); TNF-α and IL-1β (B); TGF-α (C) and IL-12 and IFN-γ (D). (*) Significantly increased levels of C5a, TNF-α, TGF-α, or IL-12 relative to pre-infused (time 0) levels ($P < 0.05$). (#) Significantly increased levels of IL-8, IL-1β, or IFN-γ relative to pre-infused (time 0) levels ($P < 0.05$).

C5a (17.64 ± 3.58 ng/mL) were observed in the milk of infected quarters within 20 and 32 h, respectively.

Initial elevations in the milk concentrations of the pro-inflammatory cytokines IL-1β and TNF-α were observed at 20 and 24 h post-infection, respectively (Fig. 5B). Milk TNF-α levels reached a peak of 11.77 ± 3.72 ng/mL and returned to baseline levels within 72 h of challenge. Maximal levels of IL-1β (0.55 ± 0.09 ng/mL), which were significantly elevated relative to pre-challenge (time 0) levels, were observed 32 h post-infection and remained elevated for up to 1 week. Augmented levels of TGF-α, a cytokine that has been reported to increase IL-1β receptor expression and to synergistically enhance the pro-inflammatory responses of IL-1β and TNF-α (Bry, 1993; Unemori et al., 1994; Subauste and Proud, 2001), were elevated within 20 h of infection and sustained for ≥2 weeks (Fig. 5C). Changes in milk TGF-α levels correlated with those of IL-1β ($r = 0.6204$), but not TNF-α ($r = 0.3393$).

Increases in IFN-γ and IL-12, two cytokines that serve to link the innate and adaptive immune systems, were detected in milk within 24 and 32 h of initial infection (Fig. 5D). IL-12 levels peaked within 32 h of infection (73.91 ± 21.96 biological units/mL) and remained elevated until 40 h after challenge. In contrast, maximal levels of IFN-γ (284.01 ± 67.13 pg/mL) were not observed until 72 h post-infection and elevations in milk IFN-γ, relative to levels at time 0, were sustained throughout the end of the study.

3.6. Changes in immunosuppressive cytokine levels during the course of P. aeruginosa intramammary infection

Resolution of infection is dependent, in part, by the downregulation of the pro-inflammatory response. Anti-inflammatory properties have been ascribed to IL-10 and members of the TGF-β family (Ayoub and Yang, 1997; Netea et al., 2003; Wahl et al., 2004). To determine whether the pro-inflammatory response elicited by intramammary infection with *P. aeruginosa* was accompanied by a compensatory anti-inflammatory response, milk levels of IL-10 and the two known TGF-β family members reported to be expressed in bovine milk, TGF-β1 and TGF-β2, were assayed by ELISA (Fig. 6). Changes in milk IL-10 levels temporally paralleled those observed for IL-12.

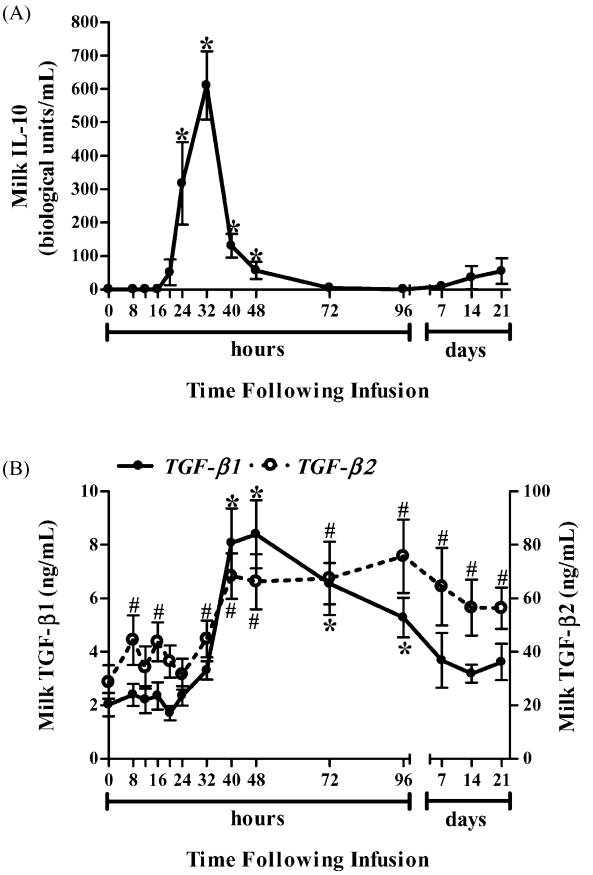

Fig. 6. Effect of *P. aeruginosa* infection on anti-inflammatory cytokine levels in milk. ELISA's were used to determine the concentrations of IL-10 (A), TGF-β1 (B), and TGF-β2 (B) in milk obtained from quarters infused with *P. aeruginosa*. (*) Significantly increased IL-10 or TGF-β1 levels compared to those in pre-infused (time 0) quarters ($P < 0.05$). (#) Significantly increased TGF-β2 levels compared to levels in pre-infused (time 0) quarters ($P < 0.05$).

Milk IL-10 levels initially increased within 24 h of infection, peaked 8 h later, and returned to pre-challenge (time 0) levels within 48 h of the initial increase (Fig. 6A). Increases in milk TGF-β1 were first detected 40 h post-challenge and remained elevated for >2 days (Fig. 6B). In contrast, heightened levels of milk TGF-β2 were observed within 8 h of challenge and, with the exception of the 24 h time point, were sustained throughout the study. Peak levels of TGF-β1 and TGF-β2 were observed 48 and 96 h after infection, respectively, reaching concentrations of 8.40 ± 1.27 ng/mL and 75.68 ± 13.68 ng/mL.

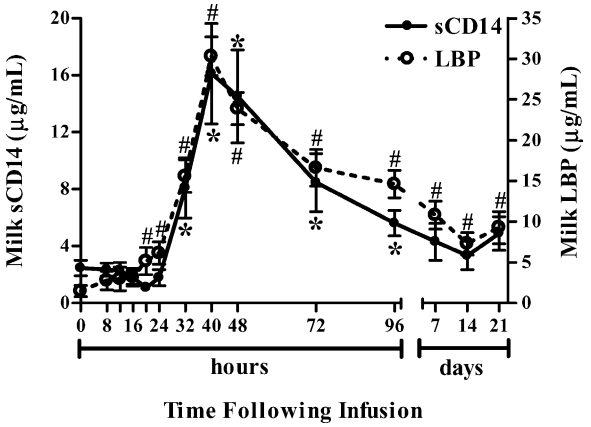

Fig. 7. Intramammary challenge with *P. aeruginosa* increases milk levels of sCD14 and LBP. Levels of sCD14 and LBP in milk obtained from quarters infused with *P. aeruginosa* were quantified by ELISA. (*, #) Significantly increased levels of sCD14 or LBP, respectively, compared to levels in pre-infused (time 0) quarters ($P < 0.05$).

3.7. Increases in milk sCD14 and LBP during *P. aeruginosa* intramammary infection

Changes in the accessory molecules sCD14 and LBP, which facilitate host recognition and neutralization of LPS, were assayed by ELISA (Fig. 7). Relative to pre-challenged (time 0) quarters, increased levels of milk LBP and sCD14 were detected within 20 and 32 h of infection, respectively. Maximal concentrations of LBP (30.32 ± 2.40 μg/mL) and sCD14 (16.10 ± 3.54 μg/mL) in milk were measured 40 h after challenge. sCD14 concentrations returned to pre-challenge levels by day 7, whereas, LBP levels remained elevated beyond day 21. Changes in LBP concentrations in milk strongly correlated ($r = 0.9566$) with those in plasma (Fig. 2B) and also correlated ($r = 0.8347$) with increases in mammary vascular permeability (Fig. 4).

4. Discussion

The successful establishment of an intramammary infection is dependent upon intrinsic virulence factors of the bacteria, itself, and the nature of the host response to the pathogen (Burvenich et al., 2003). Once a pathogen has breached host barriers, such as the keratin plug at the entrance to the teat cistern, the innate immune system represents the next line of defense in the host response to infection (Hoffmann et al., 1999). The ability of the innate immune system to recognize and respond to a broad spectrum of pathogens that may or may not have been previously encountered, as well as the rapidity of the pro-inflammatory response that ensues initial pathogen recognition, contribute to the host's control of bacterial replication. Although many aspects of innate immunity are evolutionarily conserved and even present in early phylogeny, the nature of the response to different bacterial pathogens is often variable (Hoffmann et al., 1999; Uthaisangsook et al., 2002). For example, the innate immune response to intramammary infection with *E. coli* or *Staphylococcus aureus* differs in several respects, including: (1) the degree of the febrile response; (2) the types of cytokines that are upregulated; and (3) the ability to activate milk complement (Riollet et al., 2000; Bannerman et al., 2004). Because of the importance of these differences in dictating whether a pathogen will be successfully eliminated, and since most of our understanding of the innate immune response to intramammary Gram-negative infections has been limited to studies with *E. coli*, the present investigation examined the host response to another clinically relevant Gram-negative pathogen, *P. aeruginosa*.

A critical component of innate immunity is the detection of the foreign pathogen. Pattern recognition receptors recognize conserved motifs present on foreign pathogens that are absent from host-derived tissue (Medzhitov and Janeway, 2000). sCD14 facilitates recognition of Gram-negative bacteria by binding to LPS, a component of the outer-envelope of all Gram-negative bacteria, including *P. aeruginosa*. Several studies have reported that the ability of the host to fight Gram-negative infections is impaired when levels of sCD14 are diminished (Bernheiden et al., 2001; Le Roy et al., 2001; Wenneras et al., 2001; Yang et al., 2002). In the present report, we demonstrate that intramammary infection with *P. aeruginosa* results in an increase in milk levels of sCD14 (Fig. 7). The temporal changes in sCD14 levels were similar to that previously reported during *E. coli* intramammary infection and maximal levels of sCD14 were observed in both studies 40 h after initial infection (Bannerman et al., 2004). Maximal levels

of sCD14 were slightly higher following *E. coli* challenge (Bannerman et al., 2004); however, elevated levels of sCD14 were sustained for a longer period of time following *P. aeruginosa* infection (Fig. 7). Although the origin of the increased sCD14 in milk remains unclear, it has been suggested that the elevated levels of milk sCD14 during the inflammatory response result not from leakage from the vascular compartment, but rather from shedding of neutrophil mCD14 (Lee et al., 2003a,b). Consistent with this hypothesis, increases in milk sCD14 correlated with increases in milk SCC ($r = 0.7204$), the latter of which is primarily composed of neutrophils during acute mastitis (Saad and Ostensson, 1990). Alternatively, mammary epithelial cells have been shown to upregulate sCD14, thus, providing another potential source of milk sCD14 (Labeta et al., 2000; Vidal et al., 2001).

sCD14 binding of LPS is enhanced by the accessory molecule LBP, which is derived primarily from hepatocytes and upregulated during the acute phase response to bacterial infection (Tobias et al., 1999; Schumann and Latz, 2000). Consistent with its reported induction during the acute phase response, increased levels of plasma LBP were detected following *P. aeruginosa* challenge and the temporal changes in expression were similar to that of SAA, another liver-derived acute phase response protein (Fig. 2B). Changes in milk levels of LBP (Fig. 7) strongly correlated with those in plasma and with increases in mammary vascular permeability as evidenced by increased milk BSA levels (Fig. 4). These findings suggest that much, if not all, of the increase in milk LBP is due to vascular leakage of circulating LBP. One cannot rule out a local source of LBP since IL-1β and TNF-α, which were elevated in the mammary gland during the course on *P. aeruginosa* infection (Fig. 5B), have been demonstrated to upregulate epithelial production of LBP in the intestine and the lung (Vreugdenhil et al., 1999; Dentener et al., 2000).

Host recognition of bacterial pathogens and/or their products activates intracellular signal transduction cascades that culminate in the upregulation of adhesion molecules and cytokine production, all of which contribute to leukocyte migration and the overall inflammatory response. The milk levels of two well-established pro-inflammatory cytokines, TNF-α and IL-1β, were elevated following *P. aeruginosa* infection (Fig. 5B). Both of these cytokine have been reported to contribute to the induction of several aspects of the systemic acute phase response, including the development of fever, changes in vascular permeability, and hepatic synthesis of acute phase proteins (Koj, 1996; Suffredini et al., 1999). Correspondingly, initial increases in TNF-α and IL-1β within 24 h of *P. aeruginosa* infection either preceded or were temporally coincident with maximal increases in rectal temperature (Fig. 2A), circulating levels of the acute phase proteins, SAA and LBP (Fig. 2B), and mammary gland vascular permeability (Fig. 4). Peak increases in TNF-α and IL-1β levels following *P. aeruginosa* challenge were comparable to those observed following *E. coli* infection (Bannerman et al., 2004).

In addition to their role in the induction of the acute phase response, TNF-α and IL-1β promote neutrophil recruitment to the site of infection by inducing the upregulation of vascular adhesion molecules necessary for neutrophil transendothelial migration (Pober, 1987). Neutrophil recruitment to the site of infection is further mediated by the chemoattractants IL-8 and the complement cleavage product C5a (Collins et al., 1991). Increases in milk levels of TNF-α, IL-1β, IL-8, and C5a following *P. aeruginosa* infection (Fig. 5) all preceded maximal increases in milk somatic cells (Fig. 3), the majority of which are neutrophils during the acute stages of infection (Saad and Ostensson, 1990). Relatively brief, transient increases in IL-8 versus more sustained increases in activated complement following *P. aeruginosa* challenge are similar to that previously reported following *E. coli* infection (Lee et al., 2003a,b; Bannerman et al., 2004).

Although the increases in milk levels of TNF-α, IL-1β, and IL-8 can be attributed either entirely or in part to local production by resident mammary epithelial cells and macrophages (Politis et al., 1991; Barber and Yang, 1998; Riollet et al., 2001; Alluwaimi et al., 2003), increases in C5a levels are more likely to be dependent on the influx of serum components into the gland. A previous report found that the level of complement in milk from healthy animals is relatively low (Rainard and Poutrel, 1995). In the present study, initial detection and subsequent increases in milk concentrations of C5a (Fig. 5A) paralleled increases in milk levels of BSA (Fig. 4), and changes in the concentrations of both C5a and BSA correlated with one another ($r = 0.8442$). Since increases in milk BSA

are reflective of increased mammary vascular permeability, these findings are consistent with a serum-derived influx of complement into the mammary gland, and subsequent complement activation leading to the generation of detectable C5a.

TGF-α is expressed by a variety of cell types including epithelial cells and macrophages (Madtes et al., 1988; Alison et al., 1993). Although implicated in the regulation of growth and development, as well as wound healing and maintenance of homeostasis (Kumar et al., 1995), TGF-α has also been shown to promote inflammation by upregulating the production of prostaglandins and synergistically enhancing the effects of IL-1β and TNF-α (Bry, 1993; Unemori et al., 1994; Subauste and Proud, 2001). Similar to the other pro-inflammatory cytokines including IL-8, IL-1β, and TNF-α, increased milk levels of TGF-α were observed within 24 h of *P. aeruginosa* infection (Fig. 5C). In contrast to these other pro-inflammatory cytokines, TGF-α levels were sustained for a longer period of time and did not return to baseline levels until >2 weeks after challenge. The finding that intramammary infection elicits an increase in milk TGF-α is consistent with the only other published report to study the effects of mastitis on TGF-α (Sheffield, 1997). However, in that study increases in TGF-α were measured at the mRNA level from mammary tissue, not at the protein level as reported here.

IL-12 and IFN-γ contribute to both the innate and adaptive immune responses by activating neutrophils and macrophages and promoting a T_h1-type immune response (Trinchieri, 1997). Maximal levels of both cytokines were observed 32 h after infusion of *P. aeruginosa* (Fig. 5D), and the temporal changes in these cytokines were similar to that reported during experimental mastitis induced by *E. coli* (Bannerman et al., 2004). Initial increases in IFN-γ closely paralleled those of IL-12 and are consistent with studies demonstrating that each of these cytokines can upregulate production of the other (Collins et al., 1998; Munder et al., 1998; Ma, 2001).

Although inflammation is an essential component of the host response to intramammary infection, a prolonged inflammatory response can result in injury to the epithelial lining of the mammary gland and permanently reduced milk production (Long et al., 2001; Paape et al., 2003). Resolution of the inflammatory response is mediated, in part, by the anti-inflammatory cytokine, IL-10, and members of the TGF-β family. Both IL-10 and the two TGF-β family members expressed in bovine milk, TGF-β1 and TGF-β2, moderate immune effector cell function by inhibiting pro-inflammatory cytokine production, decreasing cell proliferation, and inducing a hyporesponsive state (Ayoub and Yang, 1997; McCartney-Francis et al., 1998; Conti et al., 2003; Mocellin et al., 2004).

Consistent with the induction of a counter-regulatory anti-inflammatory response to offset the pro-inflammatory response elicited to intramammary infection with *P. aeruginosa*, increases in milk levels of IL-10, TGF-β1, and TGF-β2 were observed within 40 h of infection (Fig. 6). The temporal change in IL-10 expression was similar to that previously observed during *E. coli* intramammary infection, although the peak concentrations detected in *P. aeruginosa*-infected quarters were approximately four-fold higher (Bannerman et al., 2004). The pre-challenge (time 0) milk TGF-β1 and TGF-β2 levels of 2.03 + 0.44 ng/mL and 28.65 + 6.31 ng/mL (Fig. 6B), respectively, were comparable with those measured by others in milk samples from healthy quarters (Ginjala and Pakkanen, 1998; Pakkanen, 1998). The increased levels of TGF-β1 observed following intramammary infection with *P. aeruginosa* are consistent with reports of elevated TGF-β1 in other tissues infected by this bacterium (Kernacki et al., 1998; Rumbaugh et al., 2001). Increased levels of TGF-β have further been reported in response to Streptococcal infections (Ling et al., 2003) and during the course of septic shock induced by overwhelming infection by other bacterial pathogens (Marie et al., 1996).

5. Conclusion

The present study is the first to characterize the cytokine response to intramammary infection with *P. aeruginosa* and represents one of the most comprehensive investigations of the innate immune response to intramammary infection caused by any mastitis-causing pathogen. The data reported here establish that *P. aeruginosa* is capable of eliciting an inflammatory response characterized by the production of pro-inflammatory cytokines and chemotactic molecules, leukocyte recruitment, complement activation,

and increased levels of LPS recognition molecules. Further, the elicitation of a pro-inflammatory response to *P. aeruginosa* is accompanied by the induction of an anti-inflammatory response characterized by IL-10 and TGF-β production. The systemic and local innate immune responses to *P. aeruginosa* were similar to that reported for another Gram-negative organism, *E. coli* (Bannerman et al., 2004) and this similarity in response is most likely due to the conserved presence of immunostimulatory components found on both species of Gram-negative bacteria. Future investigations will need to focus on how the complex interplay of pro- and anti-inflammatory responses contribute to the success or failure of the mammary gland to eliminate the infection.

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